including a region bindable to the cytokine and a portion to which biotin is bound; a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and the lanthanoid metal ion are provided in an integral manner to a measurer, thereby making it possible to perform an assay for detecting the cytokine in a biological fluid sample. As necessary, the kit may further include a reference cytokine, the aforementioned various buffer solutions (in particular a buffer solution used for sample dilution and a buffer solution used for composite washing), and the like. The component items of the kit may usually be accommodated in vessels in their respectively appropriate forms, and packaged in an integral manner along with explanations or instructions for use.

The present invention makes available a novel method which is capable of detecting cytokines accurately and with high sensitivity, especially chemokines including SDF-1, in a biological fluid sample. The detection limit according to the method of the present invention may typically be about 100 pg/ml or less, preferably about 50 pg/ml or less, and more preferably

about 30 pg/ml or less, as derived under substantially the same conditions as in Example 2 described below. Similarly, a coefficient of variation (CV) for cytokine measurement may typically be less than about 10%, preferably less than about 8%, and more preferably less than about 7%, as derived under substantially the same conditions as in Example 2 described below. The recovery rate of the cytokine from a plasma sample may typically be about 70% or more, preferably about 80% or more, and more preferably about 90% or more, as derived under substantially the same conditions as in Example 6 described below. Furthermore, the fluctuations in the measured values obtained when measurements are repeated for the cytokine in plasma samples derived from the same individual under the same conditions on four or more different days may preferably be in a range of about 10 to about 20%.

As illustrated in the examples below, by utilizing an Eu^{3+} complex derived from a fluorescent compound BHHCT according to the present invention, the detection sensitivity in plasma samples was improved by two or three orders of magnitude relative to conventional methods such as ELISA and DELFIA, especially with respect to SDF-1.

It is highly important to accurately grasp the behavior of SDF-1 in vivo and reveal its physiological functions, in order to deepen the understanding of HIV-1 infections and to open up new prospects of AIDS treatment. It is evident that the present invention can make particularly significant contributions to the development and application of molecular biology concerning cytokines.

Furthermore, as illustrated in the examples below, it has been shown that, by utilizing an Eu^{3+} complex derived from a fluorescent compound BHHCT according to the present invention, measurements for cytokines other than those of the cytokine family, e.g., cytokines which exist in blood circulation as soluble factors and have biological activities in minuscule amounts and which are not only involved in various pathologies but also are already put to therapeutic applications, are possible with as high a sensitivity as that for SDF-1 and also with a good reproducibility.

EXAMPLES

Hereinafter, the present invention will be described in greater detail by way of examples. These examples are not limiting on the present invention.

Hereinafter, the present invention will be described in detail by way of examples. These examples are not limiting on the present invention.

Materials, apparatuses, and measurement conditions used in the examples are described below.

Antibodies: Anti-SDF-1 antiserum was raised by immunizing a rabbit with a multi-antigen peptide (Research Genetics, Alabama, U.S.) including residues 33-45 (RFFESHIARANVK) of human SDF-1β. The antiserum was purified by an affinity column and used. A goat polyclonal antibody to human SDF-1β was purchased from R&D Systems Inc. (Minnesota, U.S.). A human monoclonal antibody to human granulocyte-macrophage-colony stimulating factor (GM-CSF) was purchased from PharMingen (California, U.S.). A monoclonal antibody to human interleukin 2 (IL-2) was purchased from PharMingen (California, U.S.).

Chemokines: Human RANTES, human MIP-1α and β, human MDC, and human fractalkine were purchased from DIACLONE Research (France). Human IL-8 was purchased

from ENDOGEN (Massachusetts, U.S.). A commercially available ELISA kit was used for the determination of mouse IL-8 and mouse MCP-1 which were added to plasma. Mouse IL-8 was purchased from Amersham Pharmacia Biotech (Sweden), and mouse MCP-1 was purchased from PharMingen (California, U.S.). Mouse SDF-1α, mouse SDF-1β, human SDF-1α, and human SDF-1β were each donated from Genetics Institute (Massachusetts, U.S.). Human GM-CSF was purchased from PharMingen (California, U.S.). HumanIL-2 was purchased from PharMingen (California, U.S.).

Apparatuses and measurement conditions: 1420 ARVO multi-label counter from Wallac (Finland) and Amersham Pharmacia Biotech (Sweden) was used for time-resolved fluorescence measurement under the following measurement conditions: a delay time of 0.20 milliseconds (ms), a window time of 0.40 ms, and a flash rate of 1.00 ms. In order to obtain a most sensitive TR-FIA assay system, five types of microtiter plates which had been purchased from Nunc (Denmark) were examined, among which a polysorp plate produced the most sensitive fluorescence signals in the measurement of reference human SDF-1β. The order of sensitivity was as follows: White C96 maxisorp > C96 maxisorp > White C8 maxisorp > Black F16 maxisorp.

In the following experiments, White C96 polysorp microtiter plates were consistently used.

(Example 1: Preliminary study for TR-FIA)

Initially, efforts were made to identify good combinations of solid-phase-bound capture antibodies and detection antibodies which are appropriate for an ELISA-based immunoassay system for SDF-1 measurement. For this purpose, various combinations were studied from a total of five kinds including polyclonal rabbit anti-SDF-1 antibodies and polyclonal goat anti-SDF-1 antibodies. Specific detection of reference SDF-1 was observed in three combinations. However, the detection limit for SDF-1 in the ELISA assay never exceeded about 10 to 20 ng/ml. Usually, the level of SDF-1 present in plasma is much lower than such a detection limit. Thus, it was confirmed that it is virtually impossible to detect SDF-1 in plasma samples with an ELISA assay.

By employing the most preferable combinations of polyclonal antibodies that were found in the aforementioned manner, SDF-1 detection was carried out by modifying the usual TR-FIA conditions as described below.

(Example 2: TR-FIA for Reference SDF-1)

Four kinds of assay buffer solutions were prepared for TR-FIA: Buffer Solution 1 for coating a 96-well microtiter plate (0.15 M phosphate buffer (PBS) containing 0.14 M NaCl); Buffer Solution 2 for washing plates (0.05 M Tris-HCl containing 0.05% Tween20, pH 7.8); Buffer Solution 3 for washing plates (0.05 M Tris-HCl, pH 7.8); and Buffer Solution 4 for diluting protein solutions (0.05 M Tris-HCl containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl, pH 7.8).

The synthesis of BHHCT was performed following a method described in Yuan et al. ('98)(Document 5); and the preparation of a streptoavidin-bovine serum albumin (SA-BSA) conjugate and the labeling of the conjugate with BHHCT were performed following a method described in Yuan et al. ('97)(Document 4). A solution of the labeled conjugate was preserved at -20℃, and diluted 100 × with the buffer solution below (Buffer Solution 4) immediately before use.

Rabbit polyclonal anti-human SDF-1β antibody or goat polyclonal anti-human SDF-1β antibody was used as

a capture antibody. They produced similar results. A solution of the capture antibody (60 µl each), having been diluted to 10 µg/ml with Buffer Solution 1, was incubated in a well of a 96-well microtiter plate at 4℃ for 24 hours. Next, this well was washed twice with Buffer Solution 2, and once with Buffer Solution 3. The plate which has been coated with anti-SDF-1 antibody in the above manner can be preserved for at least one month at -20℃.

A reference solution of SDF-1 (50 µl) was pipetted onto the aforementioned coated plate, and incubated at 37℃ for 1 hour. After washing the plate with Buffer Solutions 2 and 3, 50 µl of a solution of biotinated goat polyclonal anti-human SDF-1 β antibody (obtained by biotinating the aforementioned goat antibody from R&D System by following usual methods), diluted 1000 × with Buffer Solution 4, was incubated in a well at 37℃ for 1 hour. After incubation, the plate was washed twice with Buffer Solution 2, and once with Buffer Solution 3, and 50 µl of a BSA-SA solution (50 µl) labeled with BHHCT-EU^{3+} was incubated in a well at 37℃ for 1 hour. The plate was washed four times with 0.05 M Tris-HCl, pH 9.1 containing 0.05% Tween20. This plate was subjected to a solid fluorescence measurement by using a 1420 ARVO multi-label

counter.

Calibration curves for the reference SDF-1 within an aqueous solution are shown in Figures 1a and 1b. The detection limit for SDF-1 by TR-FIA can be calculated from the following equation (according to Kropf et al. (Document 2):

$$3 \times [S_0] \times S_B / (S_0-B), \text{ where}$$

$[S_0]$ is a minimum concentration of the reference solution;

S_B is a standard deviation of a blank;

S_0 is a fluorescence signal intensity of the reference solution at the minimum concentration; and

B is a fluorescence signal intensity of the blank.

From the above equation, the detection limit by TR-FIA was calculated to be 30 pg/ml, which is three orders of magnitude lower than the detection limit (about 10 to 20 ng/ml) by ELISA in the aforementioned referential example. Since 50 µl of the solution is used per well, the minimum amount of SDF-1 protein detectable by TR-FIA is 1.5 pg/well.

TR-FIA was also shown to be improved with respect to measurement reproducibility. The coefficient of variation (CV) for SDF-1 detection by TR-FIA was less than 7% for a reference sample in a concentration range of 0.1 ng/ml to 1024 ng/ml. This is to be contrasted to the fact that the CV value for ELISA in the above-described referential example exceeded 10% in a concentration range of 10 ng/ml to 1000 ng/ml and that CV value for DELFIA (see the Comparative Example below) also exceeded 10% in a concentration range of 0.1 ng/ml to 1024 ng/ml.

In addition to the aforementioned solid phase fluorescence measurement, a liquid phase fluorescence measurement was also studied. Specifically, a fluorescent composite (polyclonal anti-SDF-1 antibody-SDF-1-biotinated polyclonal anti-SDF-1 antibody-BHHCT-Eu^{3+} labeled BSA-SA) formed on a solid phase by the aforementioned procedure was treated with an acidic chelated surfactant solution (a 0.1 M $NaHCO_3$ aqueous solution containing 10 μM TOPO and 0.05% SDS), thereby allowing the labeled BSA-SA conjugate to break free from the solid phase. The fluorescence intensity of the conjugate within the solution was measured by using

a 1420 ARVO multi-label counter. The SDF-1 detection sensitivity in this case was about 100 pg/ml, which is not as high as that of the aforementioned solid phase measurement.

(Example 3: Down modulation of CXCR4 by human SDF-1β)

In order to confirm the interrelationship between SDF-1 measurement values by TR-FIA according to Example 2 and the biological activity of the reference SDF-1 protein, an in-vitro down modulation of a SDF-1 receptor (CXCR4) which is induced in EL-4 cells upon binding of SDF-1 was measured.

EL-4 cells were cultured in Dulbecco-modified Eagle's medium (D' MEM), to which 10% fetal calf serum (FCS) was supplemented, under the presence or absence of human SDF-1β (1, 10, 20, 40, 100, and 1000 ng/ml). After 6 hours of incubation at 37℃, the CXCR4 on the cell surface was dyed with Fc-human SDF-1α chimeric protein and FITC-bound goat $F(ab')_2$ anti-human IgG (Southern Biotechnology Associates, Alabama, U.S.). A fluorescence intensity measurement was performed by fluorocytometry (FACSCalibur, BECTON DICKINSON, California, U.S.). The down modulation of CXCR4 was

evaluated by calculating the percentage reduction in the mean fluorescence intensity (MFI) of CXCR4 dyeing. The results are shown in Figure 1c.

From Figure 1c, it is indicated that EL-4 cells which were cultured with human SDF-1β is down modulated with respect to the CXCR4 expression in a dose-dependent manner. The results obtained were in good agreement with previous reports (Hesselgesser et al. (Document 13) and Amara et al. (Document 14)) that SDF-1α and β bind to CXCR4 with Kd values of 5-10 nM and 2.2-3.6 nM, respectively.

(Example 4: Specificity of SDF-1 measurement by TR-FIA)

In order to confirm the specificity of TR-FIA with respect to SDF-1, TR-FIA measurements similar to those described in Example 2 were taken for the following various chemokines: CC chemokines (mouse MCP-1, human MIP-1α and β, human RANTES, human MDC), CXC chemokines (human IL-8, mouse SDF-1α and mouse SDF-1β, human SDF-1α and human SDF-1β), and a CXXXC chemokine (human fractalkine). The results are shown in Figure 1d. No significant increase in the fluorescence intensity was observed in any chemokines other than SDF-1. Thus, it

was confirmed that the aforementioned TR-FIA is capable of detecting SDF-1 with a high specificity. Cross-reactivity was exhibited between human and mouse SDF-1 α and SDF-1β.

(Example 5: Preparation of plasma sample)

The plasma samples used in the following Examples were prepared from the blood of 36 healthy volunteers (Japanese) aged between 18 to 30, by using EDTA (1 mg/ml of blood) as an anticoagulant. Specifically, PBS containing 0.5 M EDTA was filled in a syringe coated with 0.1 M EDTA so that 7 µl of it would be present for every 1 ml of the collected blood. Blood was collected into this syringe, incubated at room temperature for 5 minutes, and then centrifuged at 3000 rpm for 10 minutes, thereby obtaining plasma. The plasma samples were preserved at -80℃, and diluted 10× with Buffer Solution 4 immediately before use, unless otherwise specified. It was ensured that freezing/thawing would not be repeated before the assay.

(Example 6: TR-FIA for plasma samples)

The TR-FIA as described in Example 2 was performed for the reference SDF-1 solution and the aforementioned

plasma samples (obtained from five individuals). The SDF-1 concentration in each plasma sample was calculated by comparison against a calibration curve (i.e., a line graph depicted with black circles on the left-hand side of Figure 2) which was derived from measurements of the reference solution. Furthermore, in order to confirm the accuracy of the measurements, a measurement was performed by adding 0.4 or 0.8 ng/ml of reference SDF-1 to each plasma sample, and the recovery rates were calculated.

The measured fluorescence intensities for the plasma samples having the reference SDF-1 added thereto are shown on the right-hand side of Figure 2 (under the caption "TR-FIA"). The SDF-1 concentrations and recovery rates of the plasma samples before and after the addition of the reference SDF-1 are shown in Table 1 below. It was indicated that TR-FIA makes it possible to detect SDF-1 in plasma samples as in the case of the reference solution, with high recovery rates.

(Comparative Example: DELFIA for SDF-1)

The following measurement operations of DELFIA were performed in accordance with the instructions provided by the manufacturer (Amersham Pharmacia Biotech;

hereinafter "APB"), unless otherwise specified. All washings were done by using PBS/0.05% Tween20.

A solution of rabbit anti-human SDF-1β antibody or goat anti-human SDF-1β antibody (60 µl each), having been diluted down to 10µg/ml with PBS, was adsorbed to a transparent maxisorp plate (Nunc, Denmark), incubated at 4℃ for 24 hours, and thereafter washed once. Next, in order to block non-specific binding, 180 µl of a DELFIA assay buffer solution (APB) was applied at room temperature for at least 30 minutes.

After the plate was washed three times, reference SDF-1 diluted with the DELFIA assay buffer solution, or 10× diluted plasma samples were added in an amount of 50 µl per well, and incubated at 4℃ for at least 6 hours. After the plate was washed three times, 100 µl of Eu labeled streptoavidin (APB), having been diluted down to 20 ng/ml, was added in the assay buffer solution, and incubated at room temperature for 30 minutes. After the plate was washed six times, a DELFIA sensitizing solution (APG) was added so as to allow Eu^{3+} to dissociate from the Eu-labeled antibody bound to the solid phase. After slowly shaking the microplate for 5 minutes, the

fluorescence was measured with a time-resolved fluorometer (ARVO 1420).

A calibration curve derived from measurements of the reference solution is shown on the left-hand side of Figure 2 (i.e., a line graph depicted with black squares). The detection limit which was calculated in accordance with the equation described in Example 2 be 130 pg/ml. DELFIA was able to detect SDF-1 in the reference solution, although with a lower sensitivity than by TR-FIA. However, none of the measurements of the plasma samples (from four individuals) successfully detected endogenous SDF-1. Furthermore, the recovery rates in the measurements which were taken by adding 1.0 ng/ml of reference SDF-1 to each plasma sample were about 20% or less, which is much lower than those associated with TR-FIA.

The fluorescence intensities which were measured for the plasma samples to which the reference SDF-1 was added are shown on the right-hand side of Figure 2 (under the caption "DELFIA"). The SDF-1 concentrations and recovery rates of the plasma samples before and after the addition of the reference SDF-1 are shown in Table 1. (It should be noted that the plasma samples illustrated in

Figure 2 and the data of Table 1 were all subjected to preliminary heating at 55℃ for 30 minutes).

Table. 1 Recovery rate of SDF-1 added to human plasma

	reference SDF-1 added (ng/ml)	SDF-1 measurements (ng/ml)	expected total SDF-1 (ng/ml)	recovery rate (%)
(a) TR-FIA	0	1.08	-	
	1.0	2.10	2.08	102
	0	1.53	-	
	1.0	2.48	2.53	95
	0	1.68	-	
	1.0	2.69	2.68	101
	0	1.87	-	
	1.0	2.83	2.87	96
	0	2.14	-	
	1.0	3.11	3.14	97
(b) DELFIA	0	<D.L.		
	1.0	0.20	>1.0	<20
	0	<D.L.		
	1.0	0.16	>1.0	<16
	0	<D.L.		
	1.0	0.17	>1.0	<17
	0	<D.L.		
	1.0	0.20	>1.0	<20

<D.L.: below detection limit (130 pg/ml)

(Example 7: Influences of anticoagulants and protease inhibitors)

Anticoagulants and protease inhibitors are reported to affect measurement of cytokines in human plasma samples (Thavasu et al. (Document 15)). The following experiments were conducted in order to study whether or not the SDF-1 measurement by TR-FIA is affected by such factors.

Ethylenediamine tetraacetic acid (EDTA) (1.0 mg/ml), heparin (30 IU/ml), sodium citrate (0.38%), or ethylenediamine tetraacetic acid (EDTA) (1.0 mg/ml) and aprotinin (1 μg/ml), which is a protease inhibitor, was added to plasma samples. In a manner similar to Example 2, SDF-1 was measured by TR-FIA for each sample with additions. The results are shown in Figure 3a. It was confirmed that anticoagulants and protease inhibitors do not significantly affect the plasma SDF-1 measurements by TR-FIA.

(Example 8: Influences of preliminary heating of plasma samples)

In clinical applications of SDF-1 measurement by TR-FIA, it would be necessary to inactivate HIV viruses

which may exist in blood-originated samples. Accordingly, the influences of preliminary heating of plasma samples on TR-FIA were studied.

First, in order to examine the thermal stability of SDF-1 protein, SDF-1 reference solutions were kept at 0℃ for 30 minutes; 37℃ for 30 minutes; 55℃ for 30 minutes; 70℃ for 30 minutes; or 100℃ for 1 minute, and thereafter subjected to an assay. Under the conditions of 70℃ for 30 minutes and 100℃ for 1 minute, a decrease in the detected amount was observed which was presumably due to the thermal denaturation of SDF-1. On the other hand, heating at 37 or 55℃ for 30 minutes yielded substantially the same calibration curve as those of the non-heated samples, and did not affect the detected amount of SDF-1.

Based on the above results, plasma samples from 24 individuals (see Example 5) were used, with a previous incubation at 55℃ for 30 minutes before the assay or without any heating, in order to measure SDF-1 by TR-FIA in a manner similar to Example 2. (The preliminary heating was performed before diluting the plasma samples with Buffer Solution 4). The results are shown in

Figure 3b. The preliminary heating at 55 ℃ for 30 minutes resulted in an average enhancement in fluorescence intensity of about 20%.

These results suggest the possibility that at least a portion of the SDF-1 in the plasma samples may exist in the form of multimers and/or in a bound form to a binding factor which is thermally dissociated, decomposed, etc. It is possible that the SDF-1 which exists in such multimer and/or bound forms may be inhibited from binding to an epitope.

(Example 9: Influence of dilution of plasma samples)

Previous work concerning measurement of IL-8 and MCP-1 in plasma samples (Thavasu et al. (Document 15) and Kajikawa et al. (Document 16)) has shown that the amount of chemokines present is underestimated in measurements of non-diluted samples. Accordingly, we studied the influences of dilution of plasma samples on the SDF-1 measurement by TR-FIA.

Plasma samples from 5 individuals, diluted in Buffer Solution 4 at various ratios from 1:1 to 1:20, were used to measure SDF-1 by TR-FIA in a manner similar to

Example 2. The results are shown in Figure 3c. A substantially consistent improvement in detection sensitivity was observed while the dilution ratio was increased from 1× to 10×. On the other hand, there was no improvement in the detection sensitivity when the dilution ratio was increased from 10× to 20×. Therefore, a 10× dilution (i.e., 10 parts of Buffer Solution 4 for 1 part of plasma sample) was evaluated to be the most effective condition.

(Example 10: Influences of addition of blood cells to plasma samples)

It has been reported that addition of IL-8 and MCP-1 to whole blood results in these chemokines being absorbed by the blood cells (Amara et al. (Document 14), Darbonne et al. (Document 17), and Neote et al. (Document 18)). We studied whether or not similar absorption by blood cells would be observed for SDF-1.

By subjecting 250 µl of whole blood to a microcentrifuge so as to allow the cells to deposit, plasma was obtained as a 125 µl supernatant fraction. IL-8, MCP-1 or SDF-1 was added to the 125 µl of plasma so that a predetermined final concentration was attained.

Next, the plasma in which these chemokines were added were mixed with cell pellets which were 125 µl in volume, or with 125 µl of plasma, and thereafter incubated at 37℃ for 15 minutes. Next, as for the samples in which cell pellets were mixed, cells were allowed to deposit through centrifugation and isolated. The soluble IL-8 and MCP-1 within the samples were quantified by ELISA, and the SDF-1 was quantified by TR-FIA. The results are shown in Figures **4a** to **4c**.

Most of the added IL-8 and MCP-1 were absorbed by the blood cells (Figures **4a** and **4b**). On the other hand, the reduction in SDF-1 after incubation with blood cells was less than 10% (Figure **4c**). In another experiment, SDF-1 was directly added to whole blood; after incubation, blood cells were isolated; and thereafter a TR-FIA quantification was carried out, which showed no significant difference from controls obtained by adding SDF-1 to plasma (the data are not shown). From the above, it was confirmed that SDF-1 is scarcely absorbed by blood cells.

(Example 11: TR-FIA in plasma samples--multiple detection)

For plasma samples from 36 individuals, SDF-1 was measured by TR-FIA in a manner similar to Example 2, after a preliminary heating at 55℃ for 30 minutes (see Example 7). The results are shown in Figure 5c. The SDF-1 level in human plasma had a mean value and a standard deviation of 0.85±0.26 ng/ml.

Measurements were repeatedly taken for plasma samples from the same (three) individuals, under the same conditions on four or more different days, whereby the measurement values showed fluctuations within 10 to 20%. It was shown that the plasma SDF-1 measurement by TR-FIA has a sufficiently high reliability.

(Example 12: Association of SDF-1 with IgG in plasma samples)

As for IL-8 and MCP-1, possibilities of binding or association with autoantibodies within the circulatory system are reported as another factor that may hinder immunoassays for plasma samples (Leonard et al. (Document 1) and Thavasu et al. (Document 15)). In the following manner, SDF-1 was evaluated with respect to association with IgG in plasma.

Plasma samples from 7 individuals, without heating or after a heat treatment (55℃, 30 minutes), were incubated on ice with protein G-sepharose for 30 minutes, thereby depleting IgG. The samples were centrifuged, and supernatant fractions were taken therefrom. The SDF-1 in the supernatant was measured by TR-FIA. The rate of decrease in fluorescence intensity relative to the measurement values for the plasma samples before the protein G-sepharose treatment was calculated. The results are shown in Figure 6.

In Figure 6, hatched bars and black bars represent unheated samples and heated samples, respectively. It can be seen that the unheated samples are more susceptible to influences of the protein G-sepharose treatment than the heated samples. In the unheated samples, the SDF-1 level that is measurable by TR-FIA decreased by 23 to 37% (an average of 30%) due to depletion of IgG. On the other hand, the corresponding decrease for the heated samples was 6 to 22% (an average of 15%). Thus, the effects of preliminary heating (Figure 3b) shown in Example 8 can be explained by the hypothesis that a portion of the SDF-1 in plasma samples exists in an associated form with IgG, which is dissociated through heating so as to be converted

into a soluble form that is measurable by TR-FIA.

In another experiment, no significant decrease was observed for reference SDF-1 which was added to the plasma samples even after a protein G-sepharose treatment (the data are not shown). Thus, the possibility of SDF-1 itself being adsorbed to protein G-sepharose, and the possibility of antibodies or proteins other than anti-SDF-1 IgG in the plasma samples being adsorbed to protein G-sepharose and the SDF-1 being adsorbed to such antibodies or proteins have been denied.

From the above results, it can be understood that the SDF-1 level in human plasma that is measurable by TR-FIA is very close to the physiological SDF-1 level that is actually present in blood.

(Example 13: TR-FIA for GM-CSF)

A reference solution of GM-CSF (50 μl) was subjected to a solid phase fluorescence measurement in a manner similar to Example 2, except for using anti-human GM-CSF monoclonal antibody as a capture antibody, and using biotinated anti-human GM-CSF monoclonal antibody (obtained by biotinating the aforementioned

PharMingen human antibody by following usual methods), and a calibration curve for the reference GM-CSF was produced. The results are shown in Figure 7. Furthermore, plasma samples were prepared from healthy Japanese volunteers by a method similar to that of Example 5, diluted in Buffer Solution 4 as described in Example 9, and subjected to a GM-CSF measurement by TR-FIA in a manner similar to that for the reference solution. As a result, a highly sensitive measurement was possible for GM-CSF as well, and excellent results were confirmed as far as reproducibility.

(Example 14: TR-FIA for IL-2)

A reference solution of IL-2 (50 μl) was subjected to a solid phase fluorescence measurement in a manner similar to Example 2, except for using anti-human IL-2 monoclonal antibody as a capture antibody, and using biotinated anti-human IL-2 monoclonal antibody (obtained by biotinating the aforementioned PharMingen human antibody by following usual methods), and a calibration curve for the reference IL-2 was produced. The results are shown in Figure 8. Furthermore, plasma samples were prepared from healthy Japanese volunteers by a method similar to that of Example 5, diluted in Buffer Solution 4

as described in Example 9, and subjected to a IL-2 measurement by TR-FIA in a manner similar to that for the reference solution. As a result, a highly sensitive measurement was possible for IL-2 as well, and excellent results were confirmed as far as reproducibility.

INDUSTRIAL APPLICABILITY

A time-resolved fluoroimmunoassay (TR-FIA) method which is capable of detecting cytokines, in particular chemokines including SDF-1, in a biological fluid sample with a very high sensitivity and ease of use is provided, as well as a kit for the method. The method and kit are applicable to cytokines which exist as soluble factors in blood circulation, have a biological activity in minuscule amounts, and are involved in various pathologies.

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CLAIMS

1. A time-resolved fluoroimmunoassay (TR-FIA) method for detecting a cytokine in a biological fluid sample, comprising:

forming a composite in which (a) a first antibody including a portion bound to a solid phase and a region bindable to a cytokine; (b) the cytokine; (c) a second antibody including a region bindable to the cytokine and a portion to which biotin is bound; (d) a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and (e) the lanthanoid metal ion are bound, the composite being formed on the solid phase; and

measuring fluorescence of the fluorescent structural portion which has been complexed with the lanthanoid metal ion,

wherein the fluorescent structural portion is represented by General Formula (I):

$$-R-Ar-C(=O)-CH_2-C(=O)-C_nF_{2n+1}-X \qquad (I)$$

(where R is a residue which is a functional group capable

of forming a covalent bond with a protein; Ar is a hydrocarbon group having a conjugated double bond system; n is an integer equal to or greater than 1; and X is a fluorine atom or a group represented by General Formula (II):

$$-C(=O)-CH_2-C(=O)-Ar-R- \qquad (II).$$

2. A method according to claim 1, wherein the lanthanoid metal ion is europium.

3. A method according to claim 1, wherein the fluorescent structural portion is represented by General Formula (III):

$$-R-Ar-(C(=O)-CH_2-C(=O)-C_nF_{2n+1})_2 \qquad (III)$$

(where R, Ar, and n have the same definitions as in claim 1).

4. A method according to claim 3, wherein the fluorescent structural portion is 4,4'-bis(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl)-sulpho-o-terphenyl.

5. A method according to claim 1, wherein 10 to 60 units of the fluorescent structural portion are present per molecule of streptoavidin or avidin in the conjugate.

6. A method according to claim 1, wherein the step of measuring fluorescence is performed without allowing the composite formed on the solid phase to dissociate.

7. A method according to claim 1, wherein the step of measuring fluorescence is performed after allowing the composite formed on the solid phase to dissociate.

8. A method according to claim 1, wherein the cytokine is a cytokine belonging to the chemokine family.

9. A method according to claim 8, wherein the cytokine is a CXC chemokine.

10. A method according to claim 9, wherein the cytokine is stromal cell-derived factor-1 (SDF-1).

11. A method according to claim 1, wherein the biological fluid sample is plasma or whole blood.

12. A method according to claim 1, further comprising, before the step of forming the composite, a step of diluting the biological fluid sample with a buffer solution used for sample dilution,

wherein the buffer solution used for sample dilution is 0.01 to 0.1 M tris-hydrochloric acid whose pH is 7.3 to about 8.3, the buffer solution containing 0.1 to 0.3% of bovine serum albumin, 0.05 to 0.2% of sodium azide, and 0.5 to 1.5% of sodium chloride.

13. A method according to claim 1, further comprising, before the step of forming the composite, a step of subjecting the biological fluid sample to a heat treatment under non-denaturing temperature conditions for the cytokine.

14. A method according to claim 1, further comprising, before the step of measuring fluorescence, a step of washing the composite formed on the solid phase with a buffer solution used for washing,

wherein the buffer solution used for washing the composite is 0.01 to 0.1 M tris-hydrochloric acid whose pH is 8.5 to about 9.5, the buffer solution containing

0.01 to 0.1% polyoxyethylenesorbitan monolaurate.

15. A method according to claim 1, wherein the solid phase is a microtiter plate having an IgG adsorption ability of 50 to 200 ng/cm^2.

16. A kit for a time-resolved fluoroimmunoassay (TR-FIA) method for detecting a cytokine in a biological fluid sample, comprising: a first antibody including a portion bound to a solid phase and a region bindable to a cytokine; a second antibody including a region bindable to the cytokine and a portion to which biotin is bound; a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and the lanthanoid metal ion,

wherein the fluorescent structural portion is represented by General Formula (I):

$$-R-Ar-C(=O)-CH_2-C(=O)-C_nF_{2n+1}-X \qquad (I)$$

(where R is a residue which is a functional group capable of forming a covalent bond with a protein; Ar is a hydrocarbon group having a conjugated double bond system; n is an integer equal to or greater than 1; and X is a

fluorine atom or a group represented by General Formula (II):

$$-C(=O)-CH_2-C(=O)-Ar-R- \qquad (II).$$

ABSTRACT

A method for detecting a cytokine in a biological fluid sample with a high sensitivity is provided. A time-resolved fluoroimmunoassay (TR-FIA) method including a step of forming on a solid phase a composite in which a cytokine is captured and which includes a fluorescent structural portion which has been complexed with a lanthanoid metal ion, and measuring fluorescence of the fluorescent structural portion. The composite is formed of a structure in which (a) a first antibody including a portion bound to a solid phase and a region bindable to a cytokine; (b) the cytokine; (c) a second antibody including a region bindable to the cytokine and a portion to which biotin is bound; (d) a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and (e) the lanthanoid metal ion are bound. The fluorescent structural portion is represented by General Formula (I):

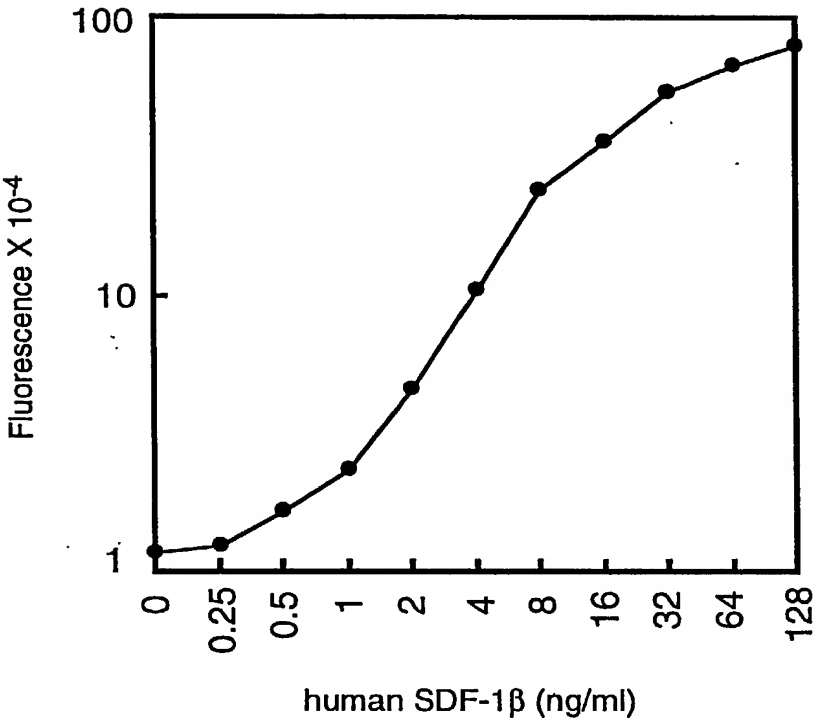
100
10
1
Fluorescence X 10-4
0
0.25
0.5
1
2
4
8
16
32
64
128
human SDF-1β (ng/ml)

Fig. 1a

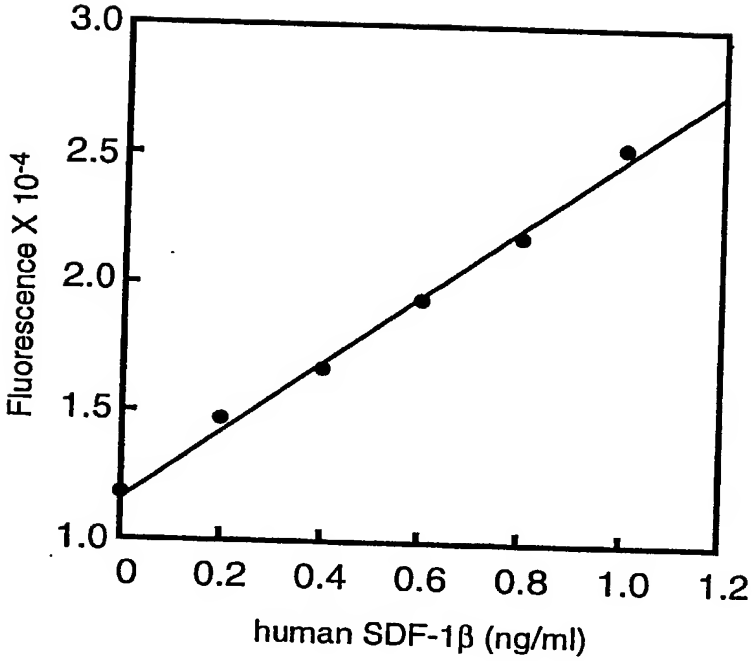
3.0
2.5
2.0
1.5
1.0
Fluorescence X 10-4
0
0.2
0.4
0.6
0.8
1.0
1.2
human SDF-1β (ng/ml)

Fig. 1b

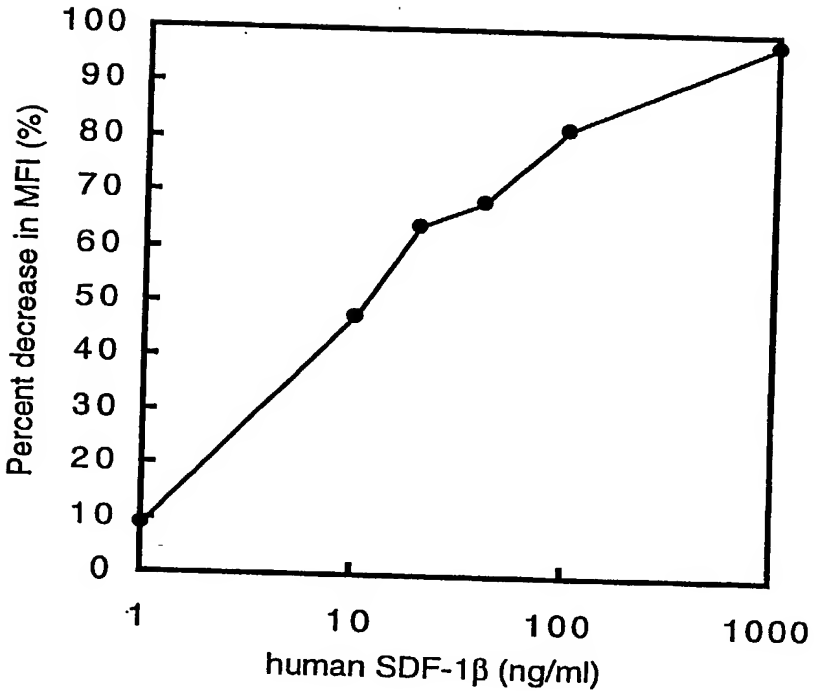
100
90
80
70
60
50
40
30
20
10
0
Percent decrease in MFI (%)
1
10
100
1000
human SDF-1β (ng/ml)

Fig. 1c

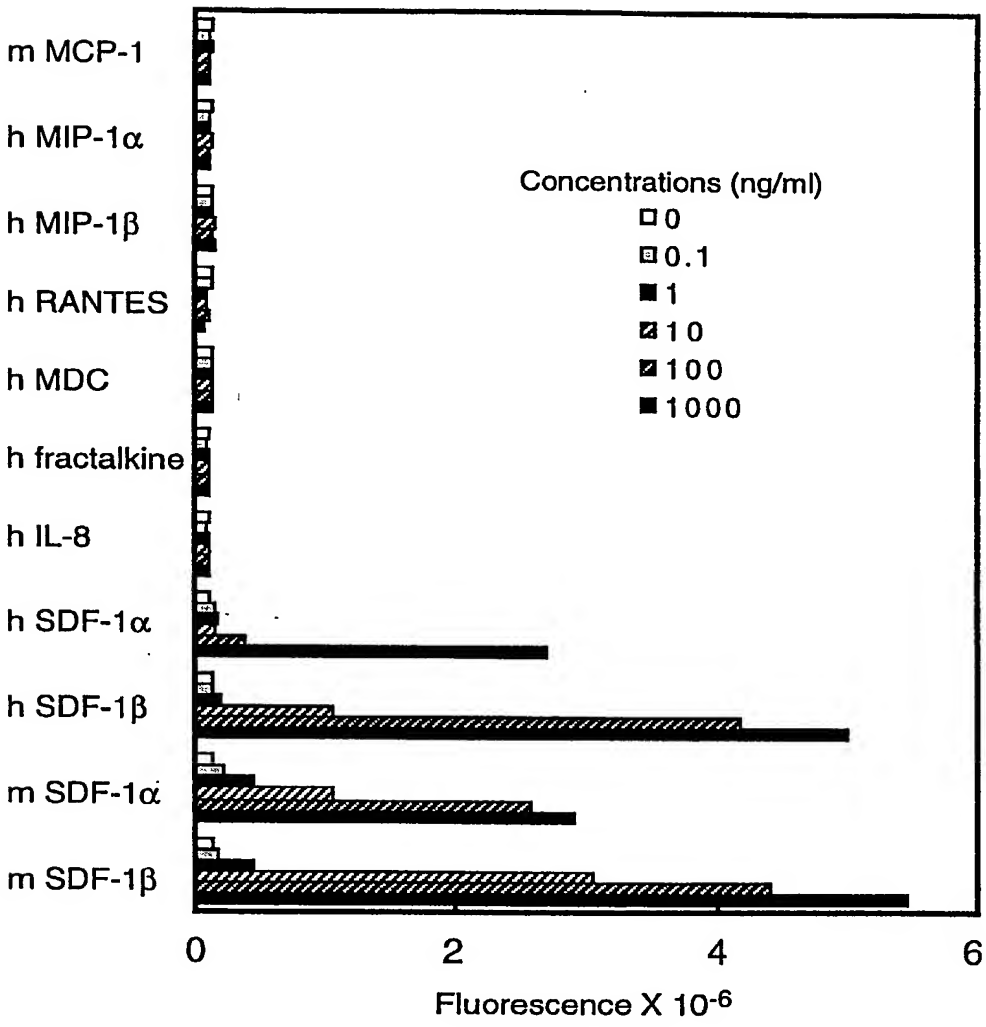
m MCP-1
h MIP-1α
h MIP-1β
h RANTES
h MDC
h fractalkine
h IL-8
h SDF-1α
h SDF-1β
m SDF-1α
m SDF-1β
Concentrations (ng/ml)
0
0.1
1
10
100
1000
0
2
4
6
Fluorescence X 10-6

Fig. 1d

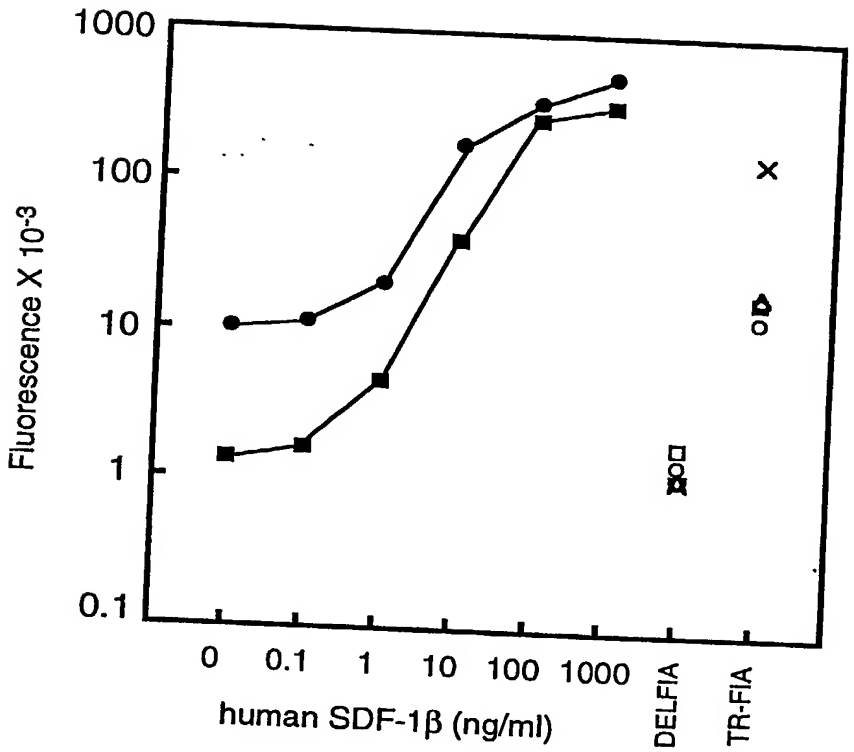
1000
100
10
1
0.1
Fluorescence X 10-3
0
0.1
1
10
100
1000
human SDF-1β (ng/ml)
DELFIA
TR-FIA

Fig. 2

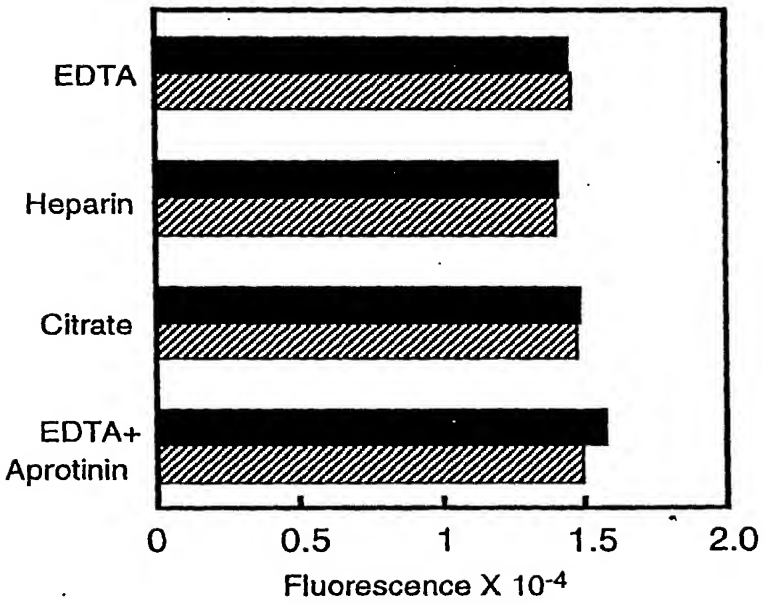
EDTA
Heparin
Citrate
EDTA+
Aprotinin
0
0.5
1
1.5
2.0
Fluorescence X 10-4

Fig. 3a

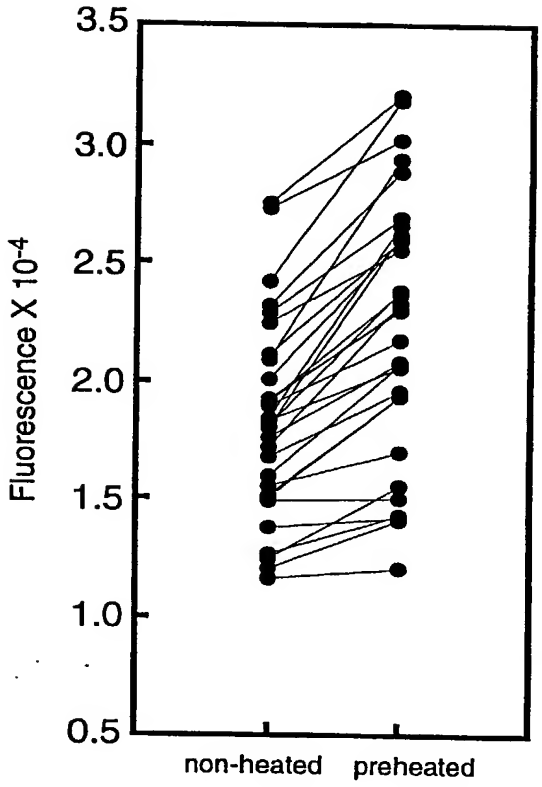

Fig. 3b

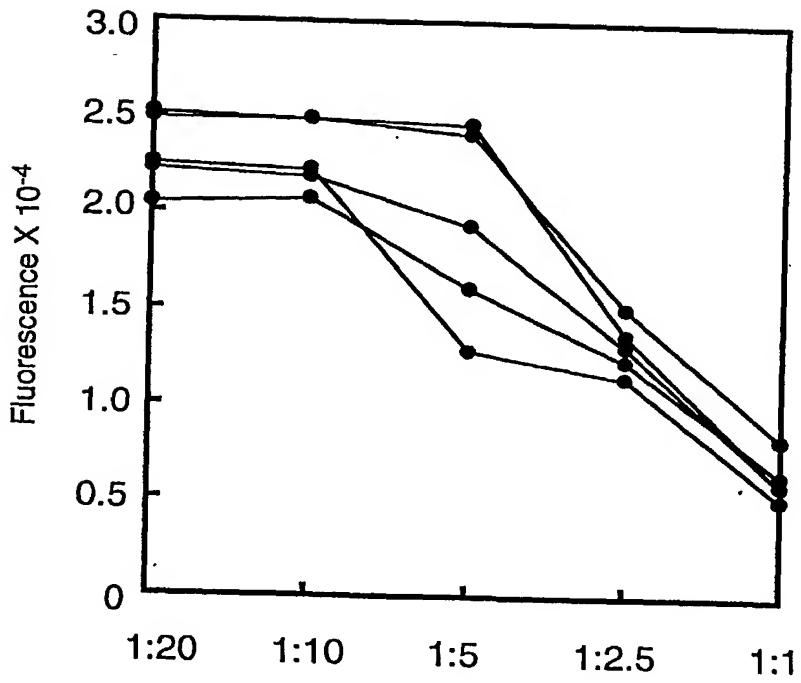

Fig. 3c

10/089776

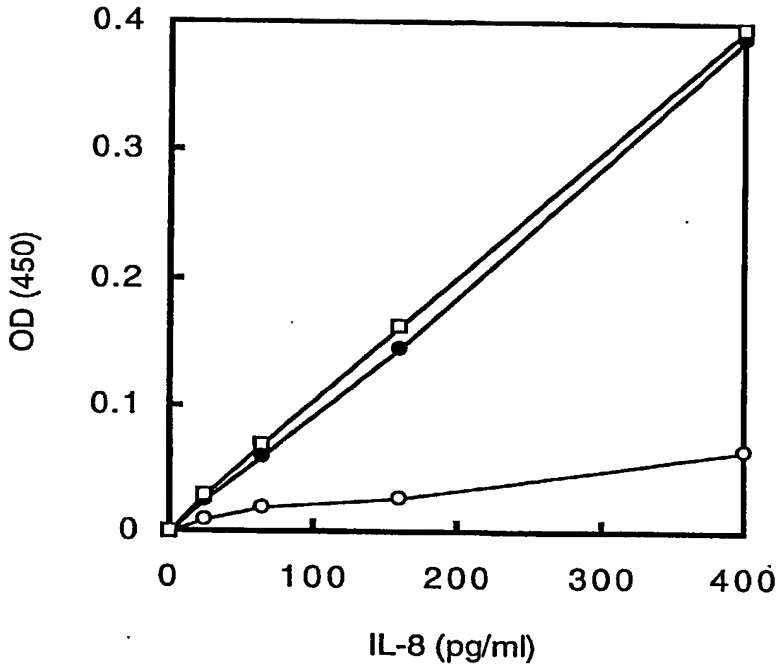

Fig. 4a

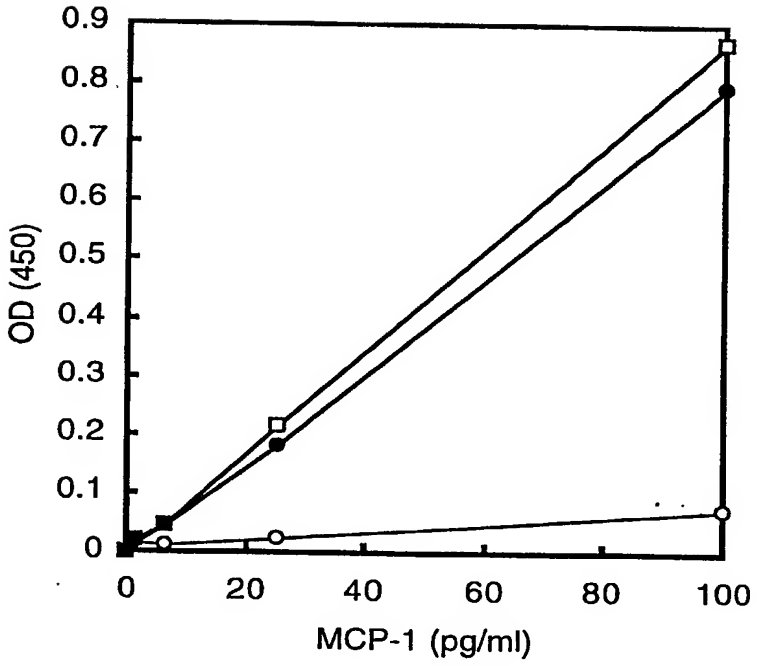

Fig. 4b

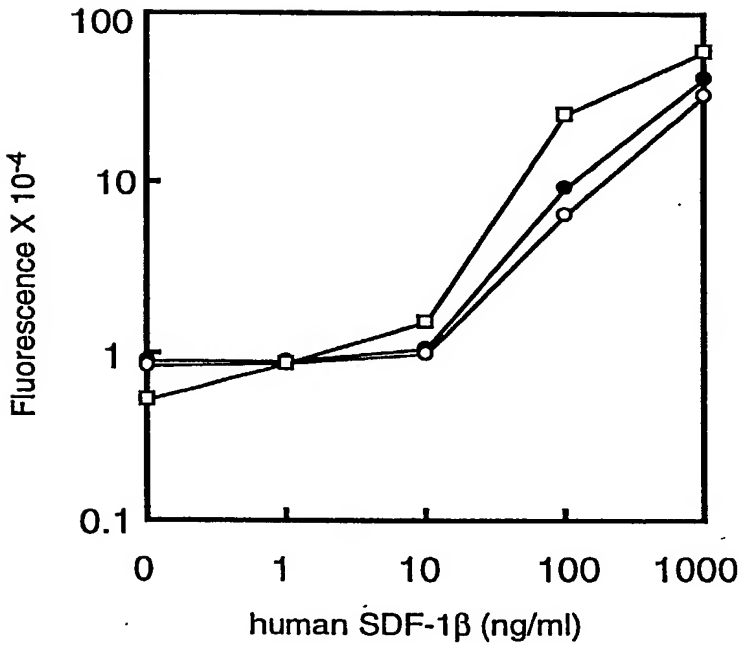

Fig. 4c

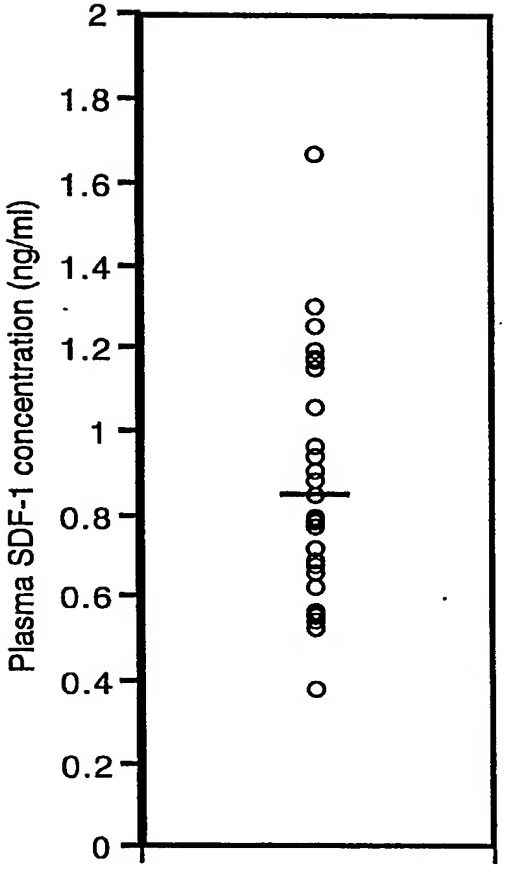

Fig. 5

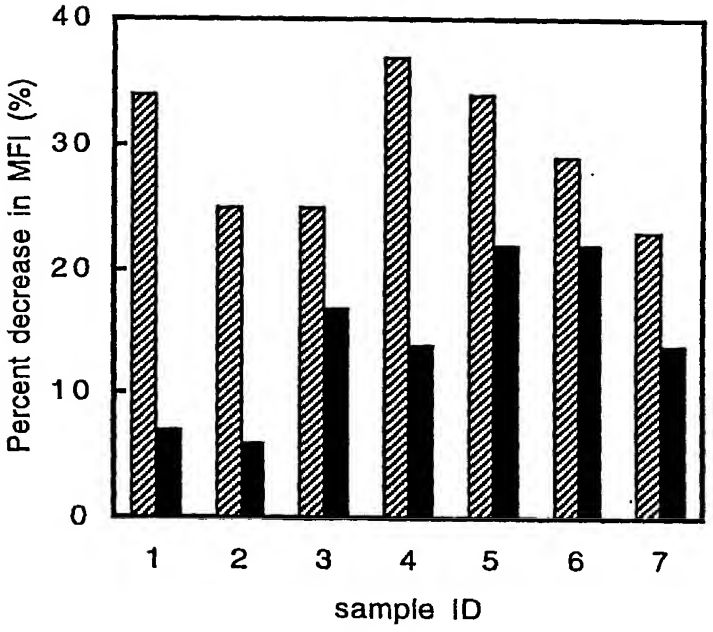

Fig. 6

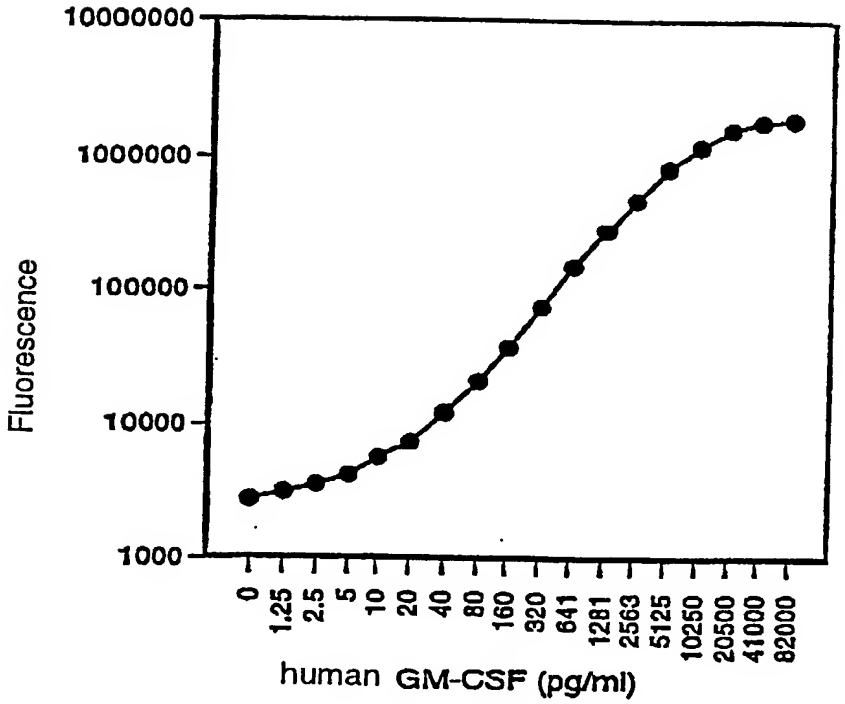
10000000
1000000
100000
10000
1000
Fluorescence
0
1.25
2.5
5
10
20
40
80
160
320
641
1281
2563
5125
10250
20500
41000
82000
human GM-CSF (pg/ml)

Fig. 7

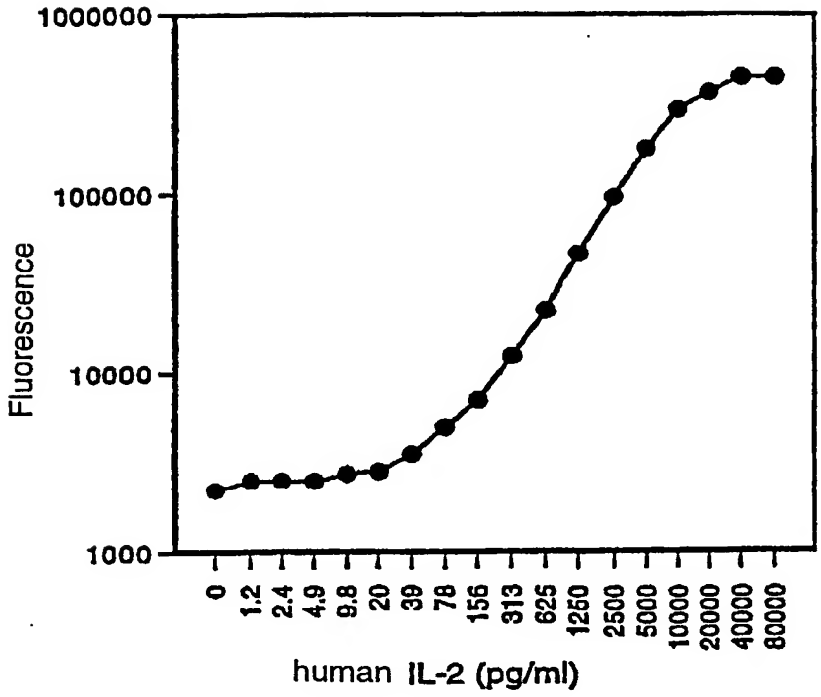
1000000
100000
10000
1000
Fluorescence
0
1.2
2.4
4.9
9.8
20
39
78
156
313
625
1250
2500
5000
10000
20000
40000
80000
human IL-2 (pg/ml)

Fig. 8

#4

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

Attorney Docket Number	29288.5600
First Named Inventor	Kei TASHIRO
COMPLETE IF KNOWN	
Application Number	10/089,776
Filing Date	March 29, 2002
Group Art Unit	To be assigned
Examiner Name	To be assigned

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HIGH SENSITIVITY IMMUNOASSAY METHOD

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 09/28/2000 as United States Application Number or PCT International Application Number PCT/JP00/06743 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above:

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	Certified Copy Attached? NO
11-277629	Japan	09/29/1999	☐	☐	☒
PCT/JP00/06743	PCT	09/28/2000	☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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DECLARATION ---- Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/JP00/06743	09/28/2000	

☐ Additional U.S. or PCT International application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transmit all business in the Patent and Trademark Office connected therewith: ☒ Customer Number 20322 → *Place Customer Number Bar Code Label here*

OR
☐ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number or Bar Code Label 20322 *OR* ☐ Correspondence address below

Name	Michael K. Kelly				
Address	SNELL & WILMER, LLP				
Address	One Arizona Center, 400 E. Van Buren Street				
City	Phoenix	State	AZ	ZIP	85004-2202
Country	USA	Telephone	602-382-6291	Fax	602-382-6070

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
1-00 Kei	TASHIRO

Inventor's Signature	Kei Tashiro					Date	3/13/02
Residence: City	Kyoto JPX	State	Kyoto	Country	Japan	Citizenship	Japan
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Post Office Address							
City	Kyoto	State	Kyoto	ZIP	603-8162	Country	Japan

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached

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ADDITIONAL INVENTOR(S) Supplemental Sheet

Page 1 of 1

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
2 - ∞ Tasuku	HONJO

Inventor's Signature	[signature]					Date	3/17/02
Residence: City	Kyoto JPX	State	Kyoto	Country	Japan	Citizenship	Japan
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Post Office Address							
City	Kyoto	State	Kyoto	ZIP	606-0001	Country	Japan

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Masaya	IKEGAWA

Inventor's Signature						Date	
Residence: City	Kyoto	State	Kyoto	Country	Japan	Citizenship	Japan
Post Office Address	401, Kitashirakawa Koporasu, 86, Kitashirakawanishimachi,						
Post Office Address	Sakyo-ku, Kyoto-shi, Kyoto Japan						
City	Kyoto	State	Kyoto	ZIP	606-8267	Country	Japan

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Kazuko	MATSUMOTO

Inventor's Signature						Date	
Residence: City	Setagaya	State	Tokyo	Country	Japan	Citizenship	Japan
Post Office Address	3-9-12-105, Daizawa, Setagaya-ku, Tokyo Japan						
Post Office Address							
City	Setagaya	State	Tokyo	ZIP	155-0032	Country	Japan

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Supplemental Sheet
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Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Tasuku			HONJO				
Inventor's Signature						**Date**	
Residence: City	Kyoto	**State**	Kyoto	**Country**	Japan	**Citizenship**	Japan
Post Office Address	19-4, Iwakuraosagicho, Sakyo-ku, Kyoto-shi, Kyoto Japan						
Post Office Address							
City	Kyoto	**State**	Kyoto	**ZIP**	606-0001	**Country**	Japan

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Masaya			IKEGAWA				
Inventor's Signature	Masaya Ikegawa					**Date**	3/13/02
Residence: City	Kyoto JPX	**State**	Kyoto	**Country**	Japan	**Citizenship**	Japan
Post Office Address	401, Kitashirakawa Koporasu, 86, Kitashirakawanishimachi,						
Post Office Address	Sakyo-ku, Kyoto-shi, Kyoto Japan						
City	Kyoto	**State**	Kyoto	**ZIP**	606-8267	**Country**	Japan

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Kazuko			MATSUMOTO				
Inventor's Signature						**Date**	
Residence: City	Setagaya	**State**	Tokyo	**Country**	Japan	**Citizenship**	Japan
Post Office Address	3-9-12-105, Daizawa, Setagaya-ku, Tokyo Japan						
Post Office Address							
City	Setagaya	**State**	Tokyo	**ZIP**	155-0032	**Country**	Japan

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Tasuku	HONJO

Inventor's Signature						Date	
Residence: City	Kyoto	State	Kyoto	Country	Japan	Citizenship	Japan
Post Office Address	19-4, Iwakuraosagicho, Sakyo-ku, Kyoto-shi, Kyoto Japan						
Post Office Address							
City	Kyoto	State	Kyoto	ZIP	606-0001	Country	Japan

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Masaya	IKEGAWA

Inventor's Signature						Date	
Residence: City	Kyoto	State	Kyoto	Country	Japan	Citizenship	Japan
Post Office Address	401, Kitashirakawa Koporasu, 86, Kitashirakawanishimachi,						
Post Office Address	Sakyo-ku, Kyoto-shi, Kyoto Japan						
City	Kyoto	State	Kyoto	ZIP	606-8267	Country	Japan

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Kazuko	MATSUMOTO

Inventor's Signature	Kazuko Matsumoto					Date	3/20/02
Residence: City	Setagaya JPX	State	Tokyo	Country	Japan	Citizenship	Japan
Post Office Address	3-9-12-105, Daizawa, Setagaya-ku, Tokyo Japan						
Post Office Address							
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